Retinal Melatonin Production: Role of Proteasomal Proteolysis in Circadian and Photic Control of Arylalkylamine *N*-Acetyltransferase

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PURPOSE. Dynamic day–night changes in melatonin synthesis are regulated by changes in the activity of serotonin *N*-acetyltransferase (arylalkylamine *N*-acetyltransferase [AA-NAT]). Similarly, a light-induced decrease in AA-NAT activity at night rapidly suppresses melatonin synthesis. The purpose of the current study was to test the hypothesis that in vivo changes of AA-NAT activity in chicken retina homogenates parallel changes in AA-NAT protein. This led to examination of the role of proteasomal proteolysis in the regulation of retinal AA-NAT activity and protein levels.

METHODS. Chickens, entrained to a 12-hour light–12-hour dark cycle, were assessed under various lighting conditions, in some cases after in vivo intravitreal administration of the protein synthesis inhibitor cycloheximide or lactacystin, an inhibitor of the 20S proteasome. Tissue homogenates were prepared, AA-NAT enzyme activity was measured, and immunoreactive protein was estimated by Western blot using an anti-chicken AA-NAT$_{1\text{-}21}$ serum.

RESULTS. The abundance of AA-NAT protein in both the retina and pineal gland exhibited a daily rhythm that was statistically indistinguishable from that of AA-NAT's activity measured in tissue homogenates. Acute exposure to light at night rapidly decreased AA-NAT protein and activity in a parallel fashion. Administration of cycloheximide at night decreased retinal AA-NAT activity in darkness and enhanced the effect of light. The light-evoked suppression of retinal AA-NAT protein and activity was blocked by intravitreal injection of lactacystin, which also was found to increase AA-NAT activity, either at night or during the daytime.

CONCLUSIONS. AA-NAT activity measured in tissue homogenates reflects the steady state level of enzyme protein. AA-NAT protein in the retina turns over rapidly, reflecting a balance of de novo synthesis and proteasomal proteolysis. The suppressive effects of light at night are due primarily to enhanced AA-NAT proteolysis. (*Invest Ophthalmol Vis Sci.* 2002;43:564–572)

Melatonin is a neurohormone produced by retinal photoreceptors and pinealocytes.[1–4] In most vertebrate species, the synthesis of melatonin in these tissues markedly increases at night, reflecting the action of lighting and circadian clocks.[5–7] Retinal melatonin acts locally to promote dark adaptation and to regulate various aspects of circadian retinal physiology.[6,8] Pineal-derived melatonin is secreted into the circulation to act at central and peripheral target tissues to control circadian physiology.[9] In addition to the well-defined circadian control of melatonin biosynthesis, mechanisms have evolved to ensure that melatonin acts strictly as a signal for darkness. As a result, unexpected light exposure at night rapidly reduces the synthesis and levels of melatonin.[10–12]

The rate of formation of melatonin is regulated in part by the activity of serotonin *N*-acetyltransferase (arylalkylamine *N*-acetyltransferase [AA-NAT]; Enzyme Commission [EC] no. 2.3.1.87), the penultimate enzyme in the melatonin biosynthetic pathway. AA-NAT activity in retina and pineal gland of most species examined undergoes robust circadian rhythms that generally parallel the changes of melatonin synthesis.[7] Recent reports indicate that a major factor regulating rhythmic and light-induced changes in activity is the steady state level of AA-NAT protein, which reflects both synthesis and degradation.[13–16] Growing evidence indicates that both processes may be subject to regulation and that there are surprising and unpredictable species-dependent differences in the relative importance of each mechanism, making analysis of each of special importance.[7]

Synthesis is a reflection of AA-NAT mRNA levels. These are relatively constant in the pineal glands of ungulates and some fish, but exhibit a nearly 10-fold increase in the avian retina and pineal[3] and a nearly 100-fold nocturnal increase in rodents.[17,18] In contrast to these species-dependent differences in the regulatory role of AA-NAT mRNA, in vitro studies of pineal glands indicate that proteasomal proteolysis may play an important role in circadian and photic regulation of AA-NAT in all species.[13–16]

The in vivo regulation of the steady state levels of AA-NAT protein in the avian retina and pineal gland has not been examined. These tissues are of special value as models of vertebrate circadian systems, because they contain complete circadian systems, including a circadian clock, a clock entrainment mechanism, and mechanisms for coupling the clock and visual transduction to melatonin biosynthesis.[3,19,20] In contrast, the mammalian pineal gland relies on a clock located in the suprachiasmatic nucleus and a photodetector located in the retina for circadian and photic input, respectively.[7]

The circadian rhythms in AA-NAT activity in the chicken retina and pineal gland in vivo reflect in part a circadian rhythm in AA-NAT mRNA levels.[3] However, this does not appear to fully explain changes in AA-NAT activity, because AA-NAT activity and mRNA do not change in parallel under all circumstances. For example, in diurnal lighting the amplitudes of the activity rhythms are higher than those of mRNA abundance, but under conditions of constant lighting they are remarkably similar.[3] In addition, light exposure at night suppresses AA-NAT activity but not AA-NAT mRNA levels.[3] These observations indicate that AA-NAT activity in

FIGURE 1. Characterization of anti-cAA-NAT$_{1-21}$ immunoreactivity. Soluble proteins of tissue homogenates were subjected to SDS-PAGE, transferred to PVDF membranes, and probed with anti-AA-NAT$_{1-21}$ antiserum or affinity-purified antibody. (**A**) The crude antiserum and the affinity-purified antibody recognized a major protein band of ~23 kDa in nighttime extracts of pineal gland. The apparent molecular weight of the protein corresponds to the molecular weight predicted from the deduced amino acid sequence of chicken AA-NAT. The intensity of the immunoreactive band was much greater in nighttime extracts than in daytime extracts. The band was absent from control blots probed with antiserum that was preincubated with AA-NAT$_{1-21}$ peptide. (**B**) The immunoreactive protein was observed in nighttime extracts of retina and pineal gland, but not in optic nerve, optic lobe, forebrain, hypothalamus, or cerebellum. The immunoreactive protein was not detected in blots of heart, liver, kidney, intestine, or skeletal muscle protein (data not shown).
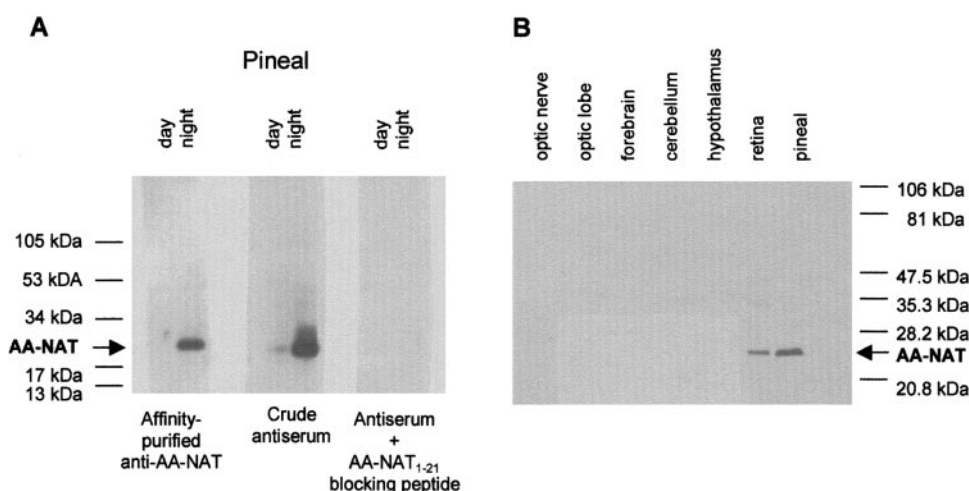
the pineal gland and retina is regulated by mechanisms other than or in addition to AA-NAT mRNA levels. A likely explanation is that activity in vivo is a reflection of the abundance of AA-NAT protein and that this is regulated by proteasomal proteolysis. This proposal is supported by the results of in vitro studies of chicken pinealocytes.[15] This was examined in the in vivo study described herein, in which chicken retinal and pineal AA-NAT activity and protein were examined. These studies provide the first evidence for light-evoked proteasomal proteolysis of AA-NAT in retinal photoreceptor cells. A preliminary report of some of these data has been presented in abstract form.[21]
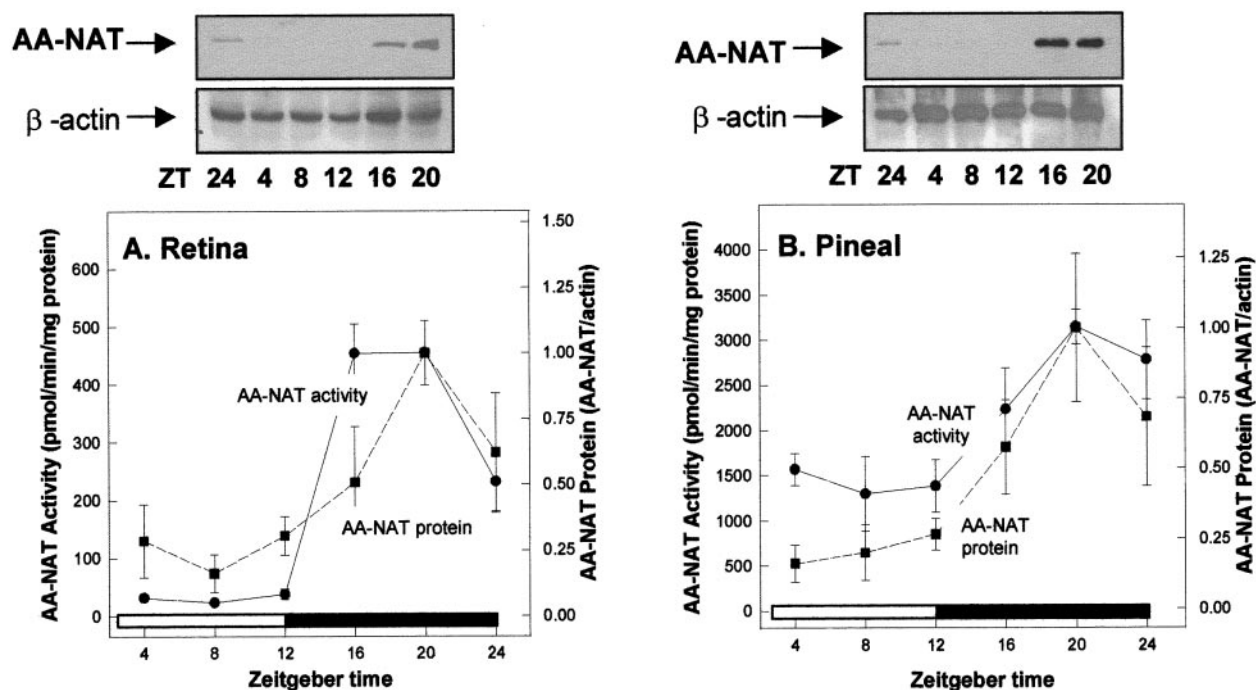

FIGURE 2. Daily rhythms of AA-NAT protein and activity in retina (**A**) and pineal gland (**B**). Subjects were housed in a 12-hour light–12-hour dark cycle, with lights on from zeitgeber time (ZT) 24/0 to 12. Here and in Figure 3, the *open bar* at the *x*-axis represents time in light, and the *filled bar* reflects time in darkness. Groups of retinas and pineal glands were sampled at the times indicated for AA-NAT immunoreactive protein and enzyme activity. Retinas and pineal glands corresponding to ZT 24 were dissected just before lights on, whereas those dissected at ZT 12 were collected in light, immediately before the time of lights off. *Top*: representative Western blot of anti-cAA-NAT$_{1-21}$ immunoreactivity and β-actin immunoreactivity. *Bottom*: AA-NAT enzyme activity and semiquantitative analysis of AA-NAT protein. AA-NAT protein is expressed as the ratio of the density of the AA-NAT–immunoreactive band to that of the actin-immunoreactive band, normalized to the ratio at ZT 20. Immunoreactive protein and enzyme activity in both tissues were rhythmic, and the rhythms of AA-NAT protein and activity for each tissue were statistically indistinguishable. $n = 4$/group (except retina ZT8, where $n = 3$). Two-factor ANOVA indicated a significant effect of time (retina, $P < 0.001$; pineal gland, $P = 0.001$) but not of measurement (retina, $P = 0.701$; pineal gland, $P = 0.237$) and no significant interaction of time and measurement (retina, $P = 0.142$; pineal gland, $P = 0.681$).

METHODS

Animals

Male White Leghorn chickens (*Gallus domesticus*) were acquired (HyLine International, Covington, GA) on the day of hatching and maintained in heated brooders in a 12-hour light–12-hour dark cycle, with lights on from zeitgeber time (ZT) 0 to ZT 12. They were used in experiments at 2 to 3 weeks of age. All manipulations of chickens and tissues (before freezing) under conditions of darkness were performed under dim red light (No. 92 filter; Eastman Kodak, Rochester, NY). Chickens were killed by decapitation. The retinas and pineal glands were rapidly dissected and frozen on dry ice. For intravitreal drug administration, chickens were anesthetized with halothane. Drugs were delivered into the vitreous cavity by injection through a 30-gauge syringe needle in a volume of 10 μL. Animal use was in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Preparation and Characterization of Anti-chicken AA-NAT(cAA-NAT)$_{1-21}$

The immunogen used to raise anti-cAA-NAT$_{1-21}$ serum (antiserum 2992) was prepared by conjugating synthetic cAA-NAT$_{1-21}$-C (Research Genetics, Huntsville, AL) to injectible maleimide-activated keyhole limpet hemocyanin (Pierce Chemical Co., Rockford, IL). The immunogen was injected into rabbits (Covance, Vienna, VA), and titer was monitored by dot blot, using cAA-NAT$_{1-21}$-C immobilized on polyvinylidene difluoride (PVDF) membrane (Immobilon-P; Millipore, Bedford, MA). Crude antiserum 2992 was immunopurified using AA-NAT$_{1-21}$-C immobilized on PVDF membrane.[22] Antibody was eluted with 0.1 M acetic acid (pH 2.85) containing 0.1% bovine serum albumin (BSA; Intergen Corp., Purchase, NY).

Sample Preparation and Assays

Retina and pineal gland were homogenized in 75 and 37.5 μL, respectively, of 0.25 M potassium phosphate (pH 6.5) containing 1.33 mM acetyl coenzyme A, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/mL leupeptin, and 1 mM NaF. Homogenates were centrifuged at 40,000*g* for 1 hour at 4°C. The supernatant fraction of each sample was used for measurement of total soluble protein,[23] AA-NAT activity[24] and AA-NAT protein by Western blot. Unless noted otherwise, 100 μg retinal protein and 10 μg pineal protein were separated by SDS-PAGE electrophoresis on 14% gels and transferred overnight at 160 mA to PVDF membranes. Blots were incubated with a 1:200 dilution of affinity-purified antiserum 2992 overnight at 4°C, followed by a 1- to 2-hour incubation with secondary horseradish peroxidase–coupled goat anti-rabbit IgG (Kirkegaard & Perry, Gaithersburg, MD). Immunoreactive proteins were detected by enhanced chemiluminescence (Amersham, Arlington Heights, IL) and analyzed by video densitometry by computer (Lynx software; Applied Imaging, Newcastle-upon-Tyne, UK). For semiquantitative analysis, blots were stripped and reprobed with a monoclonal antibody to actin (Sigma Chemical Co., St. Louis, MO); the density of the AA-NAT band was divided by that of the actin band and normalized to the ratio at either ZT 18 or ZT 20, as specified in the figure legends. In the experiment examining the effects of lactacystin, AA-NAT blots were stripped and reprobed with a polyclonal antibody to ubiquitin (Sigma Chemical Co.), before detection of actin.

Data Analysis

Data are expressed as the mean ± SEM. For statistical comparisons of AA-NAT activity with AA-NAT–actin protein ratios, activity measurements were normalized to the mean activity at ZT 18 or ZT 20, as for the AA-NAT–actin protein ratios. The normalized data were then analyzed by a two-factor ANOVA: factor 1, time of day or treatment; factor 2, measurement (activity versus protein). All other multiple comparisons were made with a one-way ANOVA with Student-Newman-Keuls test.

RESULTS

Characterization of Anti-cAA-NAT$_{1-21}$

The specificity of anti-cAA-NAT$_{1-21}$ (2992) was examined by Western blot analysis, using crude (1:20,000) or immunopurified (1:200) antiserum. Both preparations detected a single strong 23-kDa band of immunoreactive protein in extracts of chicken pineal glands obtained during the middle of the night (ZT 18; Figs. 1A, 1B). The density of the immunoreactive band was markedly lower in blots of extracts obtained in the daytime (ZT 6). A positive signal was not detected by the crude antiserum if it was first exposed to cAA-NAT$_{1-21}$-C immobilized on PVDF membrane (Fig. 1A). A 23-kDa immunoreactive protein band was also observed in extracts of retina, but not in other central nervous system tissues (Fig. 1B): heart, liver, kidney, intestine, or skeletal muscle (data not shown). These findings establish the specificity of anti-cAA-NAT$_{1-21}$ (2992) for use in immunoblot applications.

Daily Rhythms of AA-NAT Protein and Activity in Retina and Pineal Gland

AA-NAT activity was low during the daytime and high at night in retinas and pineal glands of chickens maintained in a 12-hour
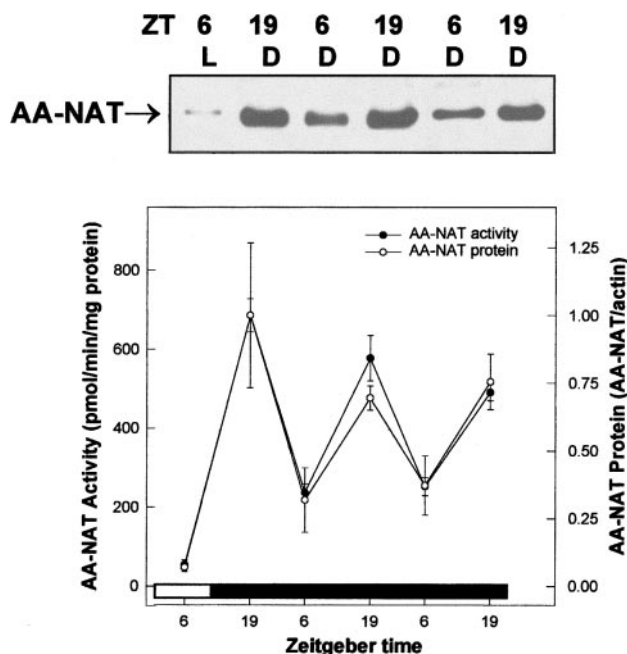

FIGURE 3. Circadian rhythm of retinal AA-NAT protein and activity. Subjects housed in a 12-hour light–12-hour dark cycle were subsequently exposed to constant darkness for 54 hours. Groups of retinas were sampled at the times indicated for AA-NAT immunoreactive protein and enzyme activity. *Top*: representative Western blot analysis of anti-cAA-NAT$_{1-21}$ immunoreactivity. *Bottom*: AA-NAT enzyme activity and semiquantitative analysis of AA-NAT protein. AA-NAT protein is expressed as the ratio of the density of the AA-NAT–immunoreactive band to that of the actin-immunoreactive band, normalized to the ratio at ZT 19 of the first day in darkness. Immunoreactive protein and enzyme activity levels followed a 24-hour rhythmic pattern, and these rhythms for each tissue were statistically indistinguishable. $n = 5$/ group. Two-factor ANOVA indicated a significant effect of time ($P < 0.001$) but not of measurement ($P = 0.694$) and no significant interaction of time and measurement ($P = 0.961$).

light–12-hour dark lighting regimen (Fig. 2). Both AA-NAT protein and activity change in parallel after a 24-hour pattern, with high levels at night. Two-factor ANOVA indicated a significant effect of time (retina, $P < 0.01$; pineal gland, $P < 0.01$) but not of measurement (protein versus activity) and no significant interaction of time and measurement. Thus, the rhythms of AA-NAT protein in both tissues are statistically indistinguishable from those of AA-NAT enzyme activity. In this experiment, as in all subsequent experiments, the density of the AA-NAT protein band in the immunoblots was normalized to that of β-actin. Representative β-actin immunoblots are shown in Figure 2. β-Actin immunoreactivity did not exhibit significant changes on a daily basis or as a function of other experimental manipulations (data not shown).

To determine whether the rhythm of AA-NAT protein levels is driven by a circadian clock, retinal AA-NAT enzyme activity and protein were measured at midday and midnight for 2 days in constant darkness (Fig. 3). AA-NAT protein level and enzyme activity showed significant circadian rhythmicity ($P < 0.01$) and fluctuated in parallel.

Effects of Light Exposure at Night on AA-NAT Protein and Activity

Exposure to bright white light in the middle of the night (ZT 18) suppressed AA-NAT protein levels and enzyme activity in a parallel fashion in both retina ($P < 0.01$) and pineal gland ($P < 0.01$; Fig. 4). Protein levels and enzyme activity were maximally suppressed after 30 minutes of light exposure. This rapid disappearance of AA-NAT protein could be due to an abrupt inhibition of AA-NAT synthesis if the protein has a constitutive short half-life, or it could be due to accelerated enzyme degradation in response to light.

Effect of Cycloheximide

In an attempt to distinguish between these possibilities, the effects of inhibition of protein synthesis were compared with those of light exposure on retinal AA-NAT protein and activity (Fig. 5). Cycloheximide (100 μg) was injected into the vitreous at ZT 17.5 under dim red light. Control eyes were injected with vehicle (H_2O). This dose of cycloheximide completely suppressed the nocturnal increase of AA-NAT activity and tryptophan hydroxylase activity in the chicken retina.[25] Thirty minutes after cycloheximide injection, AA-NAT protein and activity were reduced to 69% and 61% of the ZT 17.5 control. At this time, half of the animals were exposed to light, whereas the others remained in darkness until death at ZT 18.5. During these subsequent 30 minutes in darkness, protein level and enzyme activity continued to decrease. At ZT 18.5, the levels were 43% and 47% of control, respectively. This observation is consistent with AA-NAT's having a short half-life in darkness. Light exposure of control animals during the 30-minute period from ZT 18 to 18.5 decreased enzyme protein and activity to 24% and 47% of the ZT 17.5 control. Of special note was the observation that the combined treatments of cycloheximide and light had an additive effect, reducing enzyme protein and activity to 8% and 14% of control. Thus, in the absence of protein synthesis, light reduced AA-NAT protein levels, indicating that illumination enhances the degradation of AA-NAT.
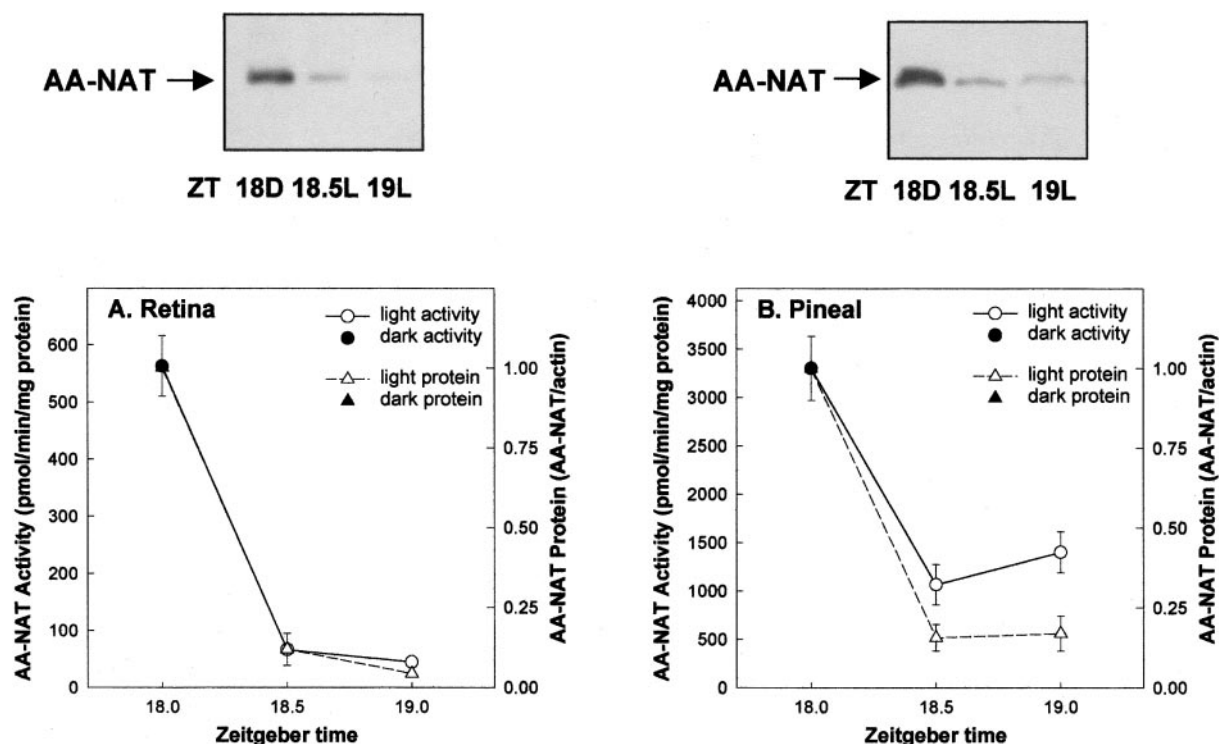

FIGURE 4. Acute light exposure at night decreased AA-NAT protein and activity. Retinas (**A**) and pineal glands (**B**) corresponding to ZT 18 were dissected at midnight in darkness (under dim red light). Other groups of animals were exposed to bright white light (~30 μW/cm^2 reflected from a white surface at cage level) beginning at ZT 18 for 0.5 or 1 hour, whereas another group remained in darkness. *Top*: representative Western blot of anti-cAA-NAT$_{1-21}$ immunoreactivity. *Bottom*: AA-NAT enzyme activity and semiquantitative analysis of AA-NAT protein. AA-NAT protein is expressed as the ratio of the density of the AA-NAT-immunoreactive band to that of the actin-immunoreactive band, normalized to the ratio at ZT 18. Light exposure significantly reduced both enzyme activity and protein. $n = 4$/group. Two-factor ANOVA: retina, time, $P < 0.001$; measurement, $P = 0.482$; time $\times$ measurement, $P = 0.849$); pineal gland, time, $P < 0.001$; measurement, $P = 0.098$; time $\times$ measurement, $P = 0.444$.

involvement of proteasomal proteolysis in the light suppression of retinal AA-NAT, chickens were injected intravitreally with the water-soluble inhibitor of the 20S proteasome lactacystin (Fig. 6). Other aqueous-insoluble inhibitors could not be tested, because they rapidly precipitated after injection into the vitreous. Lactacystin inhibited light-evoked suppression of retinal AA-NAT activity in a dose-dependent manner ($P <$ 0.001), with a maximal effect at 25 nmol/eye (Fig. 6A). At this dose, lactacystin completely inhibited the effect of light on AA-NAT enzyme activity, which remained stable in light-exposed retinas for at least 40 minutes (Fig. 6B).

Lactacystin also significantly inhibited the light-evoked decrease of AA-NAT protein immunoreactivity (Fig. 7). The effect of light on AA-NAT immunoreactivity surprisingly was only partially inhibited by lactacystin, even though the drug completely blocked the suppression of enzyme activity in the same retinas.

To confirm that lactacystin inhibited proteasomal proteolysis under these experimental conditions, the blots were re-
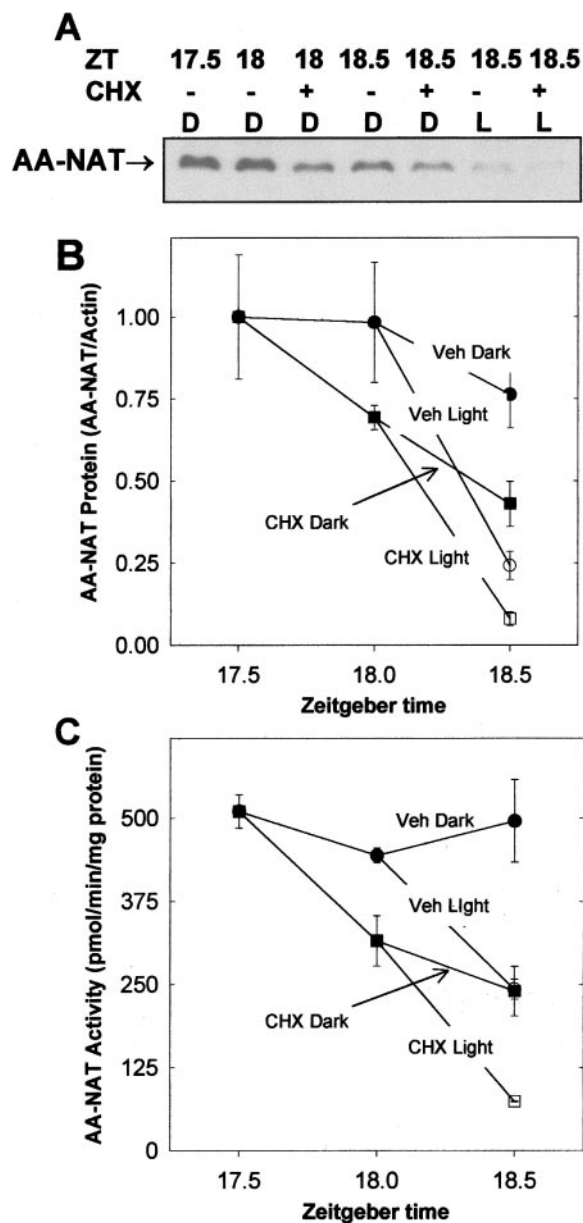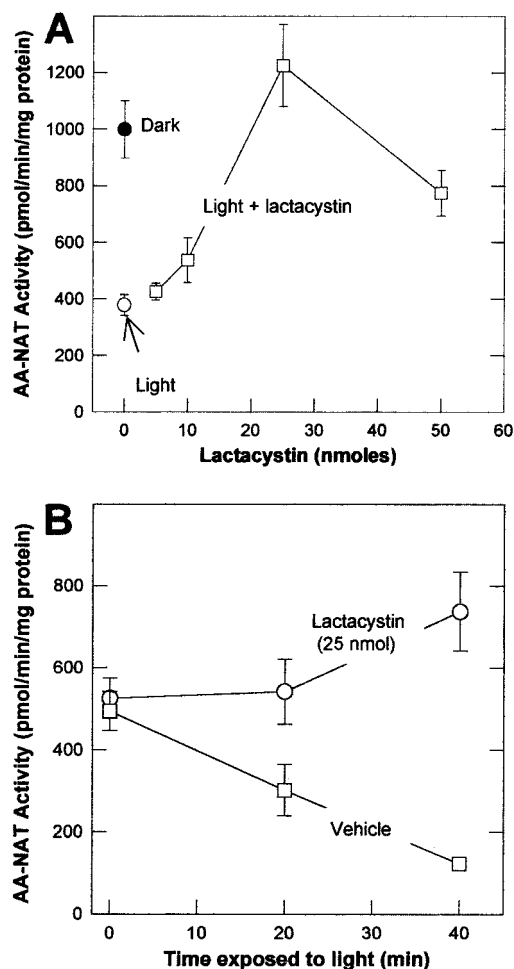

FIGURE 5. Additive effects of light and cycloheximide (CHX) on AA-NAT activity. Retinas of uninjected control eyes were dissected in darkness (D) at ZT 17.5. Anesthetized chicks were injected intravitreally with 100 μg CHX at ZT 17.5. Separate groups of chicks were injected with H$_2$O (Veh). Retinas were dissected at ZT 18 in D. Half of the chicks remained in D, whereas the others were exposed to light (L) for 0.5 hours. Retinas were dissected at ZT 18.5 and assayed for AA-NAT protein and activity. (**A**) Representative AA-NAT immunoblot. (**B**) Semiquantitative analysis of AA-NAT protein. AA-NAT protein is expressed as the ratio of the density of AA-NAT–immunoreactive band to that of the actin-immunoreactive band, normalized to the ratio at ZT 17.5. (**C**) AA-NAT enzyme activity. Light alone ($P < 0.001$) and cycloheximide alone ($P < 0.001$) decreased AA-NAT activity at ZT18.5. The effects of the two treatments were additive (light + CHX versus dark + CHX at ZT 18.5, $P < 0.001$; light + CHX versus light control at ZT 18.5, $P = 0.0012$). $n = 4$ to 5/group. Two-factor ANOVA: treatment, $P < 0.001$; measurement, $P = 0.290$; treatment × measurement, $P = 1.084$.

Effects of Lactacystin on the Light-Evoked Suppression of AA-NAT Protein and Activity

Proteasomal proteolysis has been implicated in the regulated degradation of rat pineal AA-NAT.[13] To investigate the possible

FIGURE 6. Proteasomal regulation of retinal AA-NAT activity. Lactacystin inhibited the acute inhibitory effect of light. Dark-adapted chicks were injected intravitreally at ZT 17 with lactacystin, a proteasome inhibitor, and with vehicle (contralateral eye) and were exposed to light for various times beginning at ZT 18. (**A**) Lactacystin blocked the light-evoked decrease of AA-NAT activity in a dose-dependent manner. Chicks were exposed to light for 20 minutes. Doses of 25 and 50 nmol/eye significantly attenuated the effect of light (light vehicle versus light lactacystin 25 nmol, $P < 0.001$; light vehicle versus light lactacystin 50 nmol, $P = 0.002$). $n = 4$ to 5/group. (**B**) Lactacystin, 25 nmol/eye, completely prevented the effects of 20 and 40 minutes of light exposure ($P < 0.001$). $n = 4$ to 6/group.
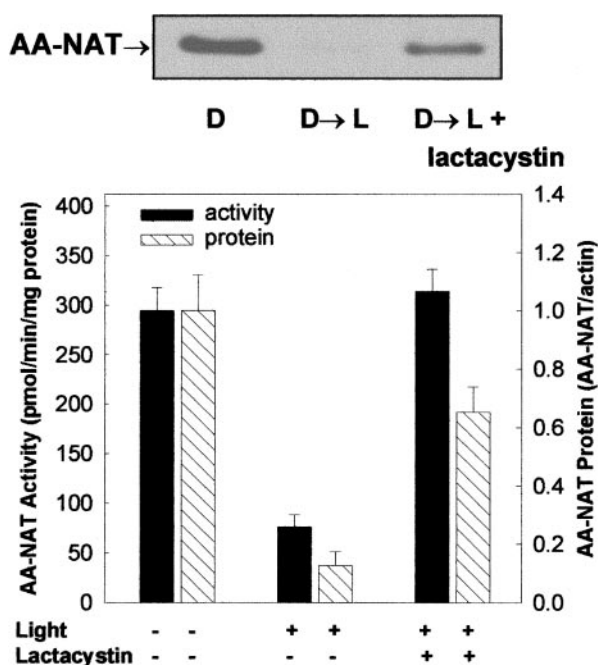
FIGURE 7. Lactacystin inhibited the light-evoked decrease of AA-NAT protein in retina. Lactacystin (25 nmol/eye) and vehicle (H$_2$O) were injected intravitreally at ZT 17. Chickens were subsequently exposed to light for 20 minutes, beginning at ZT 18. *Top*: representative Western blot for AA-NAT immunoreactive protein. *Bottom*: AA-NAT enzyme activity and protein levels, normalized to the dark control at ZT 18. Lactacystin completely prevented the light-evoked decline of AA-NAT activity and partially inhibited the light-evoked decrease of the AA-NAT–immunoreactive protein band. $n = 4$/group. Two-factor ANOVA: treatment, $P < 0.001$; measurement, $P = 0.012$; treatment × measurement, $P = 0.051$.

probed with an anti-ubiquitin antiserum (Fig. 8), because lactacystin is thought to act by blocking proteasomal processing of ubiquitinated proteins.[26] This revealed the presence of both low-molecular-weight immunoreactive ubiquitin, corresponding to free ubiquitin, and a high-molecular-weight smear of polyubiquitinated proteins. Light exposure had no significant effect on polyubiquitinated proteins (normalized ubiquitin/actin: dark 1.0 ± 0.4; dark→light 1.9 ± 0.9; $n = 4$). Lactacystin, however, elicited a large increase of ubiquitin immunoreactivity (4.7 ± 0.4; $n = 4$, $P < 0.01$), consistent with inhibited proteolysis of ubiquitinated proteins.

Effects of Lactacystin on the Daily Rhythm of AA-NAT Protein Level and Enzyme Activity

To assess the role of proteolysis in the daily rhythm of AA-NAT, lactacystin was administered intravitreally to chicks maintained in a 12-hour light–12-hour dark cycle, 1 hour before death at midnight or midday (Fig. 9). Lactacystin treatment increased both AA-NAT protein level and enzyme activity at midnight in darkness ($P < 0.01$). In contrast to the effect of lactacystin after acute light exposure at night, the increase of immunoreactive protein was equivalent in magnitude to the increase of activity. Treatment with lactacystin in the middle of the day in light increased AA-NAT activity ($P < 0.001$). The magnitude of the effect was relatively small, consistent with evidence that synthesis of AA-NAT is lower during the day, because AA-NAT mRNA is lower.

It is of interest to note that the lactacystin-induced increase in AA-NAT immunoreactive protein during the day (in light)

appeared to have a lower magnitude than that of enzyme activity, similar to the effect of lactacystin in light-exposed retinas at night. The increase of immunoreactive protein elicited by lactacystin was not statistically significant during the daytime ($P = 0.095$). Furthermore, there was a significant effect of treatment (activity versus protein) in the lactacystin-treated daytime retinas ($P < 0.001$), but not in the vehicle-treated daytime samples ($P = 0.11$).

DISCUSSION

In the results of the present study, the daily fluctuations of AA-NAT activity in chicken retina and pineal gland in vivo correlated with fluctuations of the 23-kDa immunoreactive protein AA-NAT. Furthermore, they demonstrated that the rapid suppression of AA-NAT enzyme activity resulting from light exposure at night was caused by a reduction of immunoreactive enzyme protein. These results confirm and extend the findings of Zatz et al.,[15] who demonstrated a daily rhythm of AA-NAT protein abundance in cultured chicken pinealocytes.

Zatz et al.[15] examined AA-NAT protein abundance using an immunoprecipitation assay with an antibody directed against recombinant rat glutathione-*S*-transferase (GST)-AA-NAT. AA-NAT protein was identified by autoradiography after phosphorylation by cAMP-dependent protein kinase and [^{32}P]-adenosine triphosphate (ATP). The assay detected two phosphorylated bands close to the predicted molecular weight of AA-NAT that fluctuated in parallel. In the present study, an antiserum against the N-terminal amino acid sequence of chicken AA-NAT was used that detects AA-NAT in retina and pineal gland by Western blot. In contrast to the
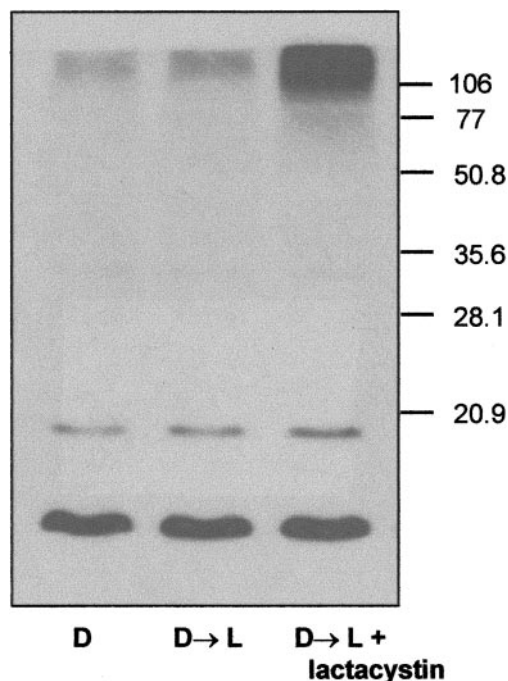

FIGURE 8. Lactacystin increases the accumulation of ubiquitin-immunoreactive proteins in retina. Lactacystin (25 nmol/eye) and vehicle (H$_2$O) were injected intravitreally at ZT 17. Chickens were subsequently exposed to light for 20 minutes, beginning at ZT 18. Retinal extracts from chicks exposed to light for 20 minutes at ZT 18 were subjected to immunoblot analysis with a polyclonal anti-ubiquitin antiserum. D, dark at ZT 18; D→L, 20-minute light exposure beginning at ZT 18. Lactacystin treatment increased high-molecular-weight immunoreactivity.
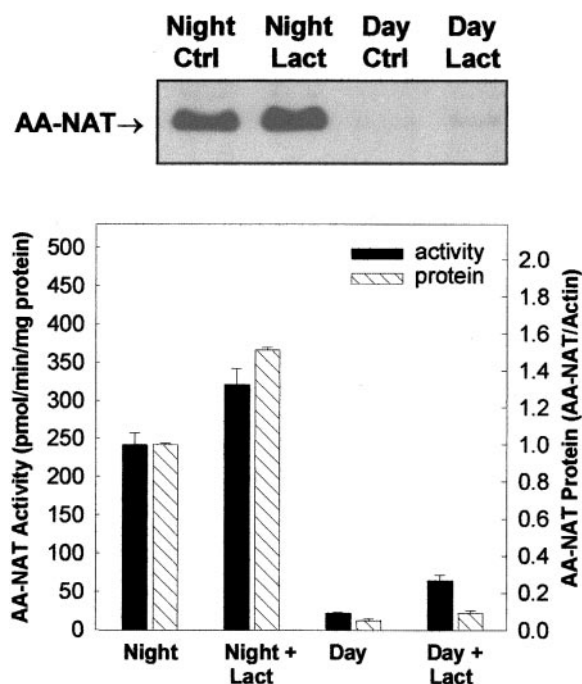
FIGURE 9. Effect of lactacystin on the daily rhythm of retinal AA-NAT protein and activity. Lactacystin (25 nmol/eye) and vehicle (H$_2$O) were injected intravitreally at ZT 5 in light and at ZT 17 in darkness. Retinas were dissected 1 hour thereafter and assayed for AA-NAT protein and activity. *Top*: representative Western blot shows anti-cAA-NAT$_{1-21}$ immunoreactivity. *Bottom*: AA-NAT enzyme activity and semiquantitative analysis of AA-NAT protein. AA-NAT protein was expressed as the ratio of the density of the AA-NAT–immunoreactive band to that of the actin-immunoreactive band, normalized to the ratio of the vehicle control at ZT 18. n = 10/group. At night, lactacystin significantly increased immunoreactive protein and enzyme activity in parallel, with no significant differences between activity and protein levels. Two-factor ANOVA: treatment, $P < 0.001$; measurement, $P = 0.427$; treatment $\times$ measurement, $P = 0.429$. During daytime, lactacystin significantly increased AA-NAT activity ($P < 0.001$), but not that of protein ($P = 0.095$). Two-factor ANOVA: treatment, $P < 0.001$; measurement, $P < 0.001$; treatment $\times$ measurement, $P < 0.001$.

assay of Zatz et al.,[15] anti-cAA-NAT$_{1-21}$ detected only a single major protein band of 23 kDa in pineal extracts. The reason for this discrepancy is unclear. AA-NAT contains two consensus phosphorylation sites for cyclic nucleotide-dependent protein kinases.[3,7,27] Thus, the two radioactive protein bands observed in the earlier study may reflect mono- and diphosphorylated forms of the enzyme. Alternatively, the bands may represent intact and partially degraded enzyme protein, because the anti-cAA-NAT$_{1-21}$ serum used in the present study would not detect a partially degraded protein if the N-terminal 21 residues had been deleted. It is also possible that other posttranslational modifications of this sequence influence the immunodetection of AA-NAT.

The rhythm of chicken AA-NAT activity appears to reflect rhythmic changes in the steady state levels of enzyme molecules, as indicated by the parallel changes of enzyme protein and activity observed in the present study. Two processes appear to contribute to the observed rhythm in AA-NAT protein. As discussed in the following paragraph, one is the clock-driven rhythm in AA-NAT mRNA, which is translated into a rhythm in synthesis. The second is enzyme degradation, which is regulated by light.

Unexpected light exposure at night rapidly suppresses AA-NAT activity and melatonin levels in chicken retina and

pineal gland.[12] In the current study, this rapid suppression was caused by a decrease of enzyme protein. The suppression of enzyme activity under these conditions was not accompanied by a comparable decline in AA-NAT mRNA abundance,[3] suggesting a posttranscriptional site of regulation. This is supported by the evidence that AA-NAT has a constitutively high turnover rate in darkness, as indicated by the finding that inhibition of protein synthesis at night in darkness caused AA-NAT activity and protein to decrease. It is not clear, however, whether physiological regulation by light involves inhibition of protein synthesis. This is not unreasonable to consider, because the decrease in AA-NAT activity and protein due to light treatment was similar to that produced by inhibition of protein synthesis. However, it was also found that cycloheximide treatment markedly enhanced the effects of light, indicating that light does not act exclusively by inhibiting protein synthesis. but rather it acts, at least to a large degree, by accelerating AA-NAT degradation. The possibility that light also inhibits translation remains open to further investigation.

The results of several experiments and our understanding of the regulation of AA-NAT in other vertebrate systems are consistent with the hypothesis that photic regulation of chick AA-NAT protein is due to cAMP control of AA-NAT proteolysis. This is supported by several observations. First, photoreceptor cAMP is regulated by light exposure, which decreases levels of this second messenger.[28-31] Second, cAMP inhibits the decline of AA-NAT activity in cycloheximide-treated photoreceptor cells.[32] Third, the cAMP protagonist forskolin increases AA-NAT protein and activity in chick pinealocytes,[15] apparently due to reduced destruction of AA-NAT. Fourth, as discussed earlier, when protein synthesis is blocked, AA-NAT protein disappears, and forskolin can prevent this,[32] presumably by elevating cAMP. These observations lead us to conclude that light and darkness act through cAMP to regulate AA-NAT protein. Other observations indicate that cAMP is probably acting through phosphorylation of the highly conserved PKA sites in AA-NAT, at least one of which is known to be phosphorylated at night in the ovine pineal gland.[33]

Proteasomal proteolysis has been implicated in the regulation of AA-NAT protein turnover in pineal gland. Proteasome inhibitors increase AA-NAT protein levels of cultured rat pineal cells and prevent the reduction in protein levels caused by blocking β-adrenergic receptors.[13] Similarly, treatment with proteasome inhibitors increased AA-NAT protein levels and enzyme activity in cultured chick pinealocytes.[15] In the present study, lactacystin, an inhibitor of the 20S proteasome, significantly inhibited the light-evoked suppression of retinal AA-NAT protein abundance and enzyme activity in vivo. Thus, light may stimulate proteasomal degradation of AA-NAT in photoreceptor cells. Inhibition of proteasomal degradation by treatment with lactacystin prevented the light-evoked suppression of AA-NAT activity but did not increase it significantly above the levels in dark-adapted retinas. The surprising observation that light treatment partially decreased AA-NAT protein in the presence of lactacystin indicates that under certain experimental conditions AA-NAT protein and activity do not change in parallel.

Lactacystin also increased AA-NAT protein and activity at midnight in darkness, consistent with the hypothesis that AA-NAT is being synthesized and destroyed continually in darkness. The effect of lactacystin on AA-NAT activity and protein in darkness was similar; both increased in parallel. In contrast to this relationship, lactacystin treatment in the light caused AA-NAT activity to increase more than that of immunoreactive protein. This provides further evidence that AA-NAT activity and protein do not change in parallel under all conditions.

The reason for the loss of parallelism is not clear but could involve a posttranslational modification of the enzyme protein that changes immunoreactivity without changing catalytic activity. For example, light exposure may lead to structural modification that does not change activity but increases targeting to the proteasome through modifications that reduce immunoreactivity. This would result in an apparently greater preservation of activity than of immunoreactive protein. Many proteins targeted for proteasomal degradation are ubiquitinated at lysine residues near the N-terminal,[26] and ubiquitin conjugation and degradation in rat retina are greater under light-adapted than dark-adapted conditions.[34] Ubiquitination at Lys-10 of AA-NAT may block immunoreaction with our antibody, which was raised against N-terminal amino acids 1-21. It is also possible that the immunoreactive band of AA-NAT is a reflection of both denatured and active protein and that both are always present in the cell. Accordingly, lactacystin may have a more dramatic effect on enzyme activity, because the assay measures only active protein, whereas the Western blot assay detects both active and denatured protein.

Recently, AA-NAT was shown to bind to the ubiquitous signaling protein 14-3-3.[35] The binding of AA-NAT by 14-3-3 apparently modulates its enzyme activity by stabilizing a region of the enzyme involved in substrate binding.[35] Although the role of this interaction in photoreceptors remains to be investigated, it seems reasonable to suspect that it may play a role in the activity and/or stability of AA-NAT in the retina, and as a result, in melatonin production in this tissue.

In conclusion, proteasomal proteolysis plays a prominent role in the physiological regulation of retinal AA-NAT activity. The rapid suppressive effect of light on AA-NAT activity and melatonin biosynthesis is primarily a reflection of enhanced enzyme proteolysis.

Acknowledgments

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